Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts

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Accelerated atherosclerosis in patients with diabetes is a major cause of their morbidity and mortality, and it is unresponsive to therapy aimed at restoring relative euglycemia. In hyperglycemia, nonenzymatic glycation and oxidation of proteins and lipids results in the accumulation of irreversibly formed advanced glycation endproducts. These advanced glycation endproducts engage their receptor in cells of the blood vessel wall, thereby activating mechanisms linked to the development of vascular lesions. We report here a model of accelerated and advanced atherosclerosis in diabetic mice deficient for apolipoprotein E. Treatment of these mice with the soluble extracellular domain of the receptor for advanced glycation endproducts completely suppressed diabetic atherosclerosis in a glycemia- and lipid-independent manner. These findings indicate interaction between the advanced glycation endproducts and their receptor is involved in the development of accelerated atherosclerosis in diabetes, and identify this receptor as a new therapeutic target in diabetic macrovascular disease.

The factors underlying accelerated atherosclerosis in diabetes extend beyond dyslipidemia, hypertension and obesity. Even after correction of these typical risk factors, patients with diabetes continue to experience increased atherosclerotic vascular disease[1,2]. The Diabetes Control and Complications Trial Research Group studies have shown that aggressive therapeutic efforts aimed at restoring relative euglycemia diminish pathology in the microvasculature. However, there was no significant reduction in the incidence of macrovascular disease in patients with rigorous control of glycemia[3,4]. Although many factors probably underlie these findings, these data indicated that delineation of the sustained, irreversible consequences of even sporadically elevated blood glucose might be essential to design targeted, effective therapy for this disorder. In diabetes, hyperglycemia drives nonenzymatic glycation and oxidation of proteins and lipids, enhancing irreversible formation of advanced glycation endproducts (AGEs); these processes are especially favored in the diabetic, hyperlipidemic environment[5,6]. Thus, accumulation of pre-formed AGEs in diabetic blood vessels may be involved in the pathogenesis of diabetic vascular disease. Furthermore, AGE engagement of the receptor for AGE (RAGE) (refs. 7,8) in cells of the vessel wall perturbs vascular homeostatic functions, activating mechanisms linked to development of vascular lesions[9–13].

To investigate the molecular mechanisms underlying accelerated diabetic atherosclerosis, we developed a murine model for this. Accelerated formation of complex atherosclerotic lesions in diabetic rodents has been difficult to achieve, mostly because of their inherent resistance to the development of macrovascular disease, even on fat-enriched diets[14]. Indeed, C57BL/6 and BALB/c mice made diabetic with streptozotocin (stz) and fed an atherogenic diet developed lesions limited to fatty streaks[15].

Here, we studied mice deficient in apolipoprotein E (apoE-null)(refs. 16,17); in these mice, severe hypercholesterolemia despite eating a normal chow diet causes atherosclerosis. To determine whether induction of diabetes would accelerate the incidence and progression of vascular lesions, mice were made diabetic using streptozotocin. We demonstrate here a model of accelerated and complex atherosclerosis in these diabetic apoE-null mice. Treatment of these mice with a truncated, soluble form of RAGE (sRAGE), which includes the extracellular ligand-binding domain, suppressed the development of accelerated diabetic atherosclerosis in a dose-dependent manner. The beneficial effects of sRAGE were independent of experimentally-induced alterations in glycemia, insulinemia and lipid profile. These findings indicate AGE–RAGE interaction is involved in the pathogenesis of accelerated atherosclerosis in diabetes, and identify RAGE as a new target for therapeutic intervention in diabetic macrovascular disease.

Atherosclerosis in diabetic mice deficient in apoE

Male, 6-week-old apoE-null mice were treated with streptozotocin[18] (stz); hyperglycemia (serum glucose concentration greater than 350 mg/dl), beginning within seven days of administration of stz, was documented in more than 95% of these mice. Serum glucose in stz-treated mice was 350–500 mg/dl, compared with 130–160 mg/dl in citrate-treated control mice ($P < 0.005$). Stz-treated mice with serum glucose >350 mg/dl were classified as diabetic. Mean levels of glycosylated hemoglobin, which represents an early and reversible stage of non-enzymatic glycation[19], in nondiabetic controls were about 34% those in mice treated with stz after six weeks of diabetes ($3.7 \pm 0.28\%$ ($n = 11$) versus $10.9 \pm 0.57\%$ ($n = 11$); $P < 0.0005$), consistent with persistent hyperglycemia in the latter group. Stz-induced diabetes resulted from relative insulin deficiency, as shown by lev-

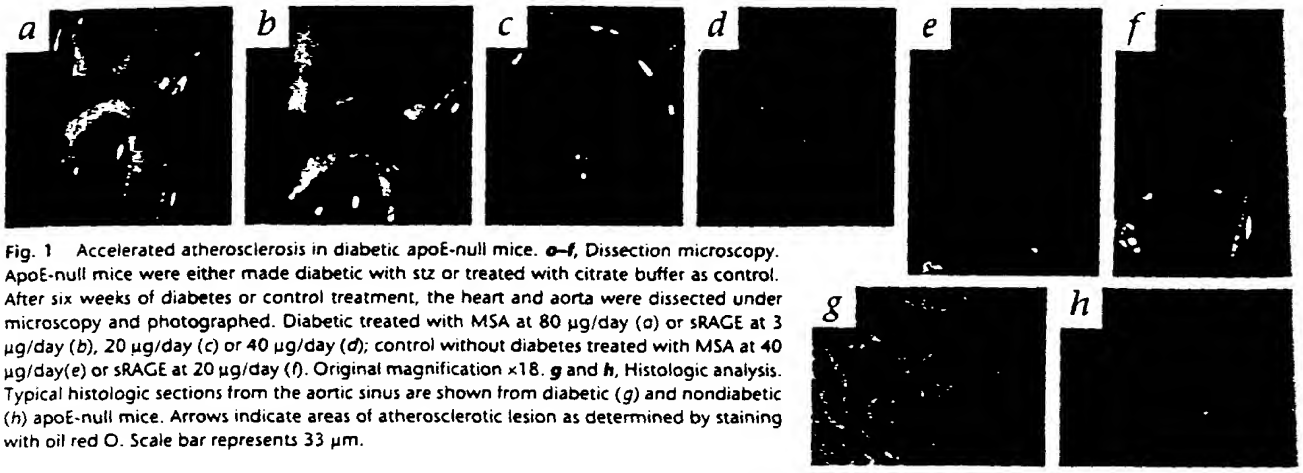

Fig. 1 Accelerated atherosclerosis in diabetic apoE-null mice. ***a–f***, Dissection microscopy. ApoE-null mice were either made diabetic with stz or treated with citrate buffer as control. After six weeks of diabetes or control treatment, the heart and aorta were dissected under microscopy and photographed. Diabetic treated with MSA at 80 μg/day (*a*) or sRAGE at 3 μg/day (*b*), 20 μg/day (*c*) or 40 μg/day (*d*); control without diabetes treated with MSA at 40 μg/day(*e*) or sRAGE at 20 μg/day (*f*). Original magnification ×18. ***g*** and ***h***, Histologic analysis. Typical histologic sections from the aortic sinus are shown from diabetic (*g*) and nondiabetic (*h*) apoE-null mice. Arrows indicate areas of atherosclerotic lesion as determined by staining with oil red O. Scale bar represents 33 μm.

els of serum insulin in diabetic mice 62% those in nondiabetic controls (0.62 ± 0.03 ng/ml (n = 5) versus 1.0 ± 0.06 ng/ml (n = 5); P = 0.002). Stz-treated mice did not demonstrate ketonuria (data not shown).

ApoE-null mice on a normal chow diet usually first manifest foam-cell lesions at eight weeks of age, and develop more advanced, complex lesions after fifteen weeks of age[20]. After six weeks of diabetes (at about 13–14 weeks of age), diabetic apoE-null mice had discrete lesions at major branch points in the thoracic aorta and at the arch of the aorta, sites known to be predisposed to such lesions (Fig. 1*a*). In distinct contrast, age-matched, euglycemic apoE-null mice did not have lesions in the proximal aorta (Fig. 1*e*). Consistent with these observations, quantitative morphometric analysis[21] showed an approximately 5.3-fold increase in mean lesion area at the level of the aortic sinus in diabetic apoE-null mice compared with that in nondiabetic apoE-null mice. Mean atherosclerotic lesion area in diabetic apoE-null mice (n = 14) was 169,415 ± 13,145.4 μm²; in contrast, in nondiabetic apoE-null mice (n = 8), mean atherosclerotic lesion area was 31,718 ± 7,749.6 μm²; P < 0.00005. Histologic analysis at the aortic sinus of euglycemic apoE-null mice showed typical fatty streaks, visualized with oil red O (Fig. 1*h*). However, diabetic apoE-null mice had larger, more advanced fibrous plaques, with a propensity for cap formation (Fig. 1*g*).

Mechanisms underlying accelerated atherosclerosis

Many alterations in lipid profile may occur in patients with diabetes, including hypertriglyceridemia and diminished plasma levels of HDL. In our stz–apoE-null model, diabetes was followed by a time-dependent increase in plasma total cholesterol. After six weeks of diabetes, diabetic apoE-null mice had an approximately twofold increase in cholesterol compared with that in nondiabetic mice (1,359 ± 143 versus 500 ± 25 mg/dl; P < 0.005), whereas plasma triglyceride levels were similar for these groups (147 ± 36 versus 133 ± 15 mg/dl; P = 0.30). Fractionation of plasma cholesterol by density gradient ultracentrifugation demonstrated increased chylomicrons/VLDL (about 2.6-fold) and IDL/LDL (about 2.5-fold) in age-matched diabetic apoE-null versus euglycemic apoE-null mice, whereas HDL was more modestly altered (levels in diabetic mice 150% those in control mice) (Fig. 2*a*, left). In contrast, there were minimal-to-no differences between these groups in plasma triglycerides (Fig. 2*a*, right). FPLC gel filtration, to fractionate pooled plasma, confirmed the increase in cholesterol in diabetic mice, whereas triglyceride were shown to remain unchanged (data not shown).

Diabetic apoE-null mice (n = 14) had increased formation o AGEs compared with that in euglycemic apoE-null controls (n = 6), as shown by an approximately 2.3-fold increase in AGE-immunoreactive epitopes in acid-soluble material extracted from kidney tissue after six weeks of diabetes (11.85 ± 1.1 ng/ml versus 5.2 ± 0.92 ng/ml AGE BSA equivalents; P < 0.0002). Similarly, diabetic apoE-null mice (n = 6) had an approximately 2.5-fold increase in plasma AGEs compared with those in nondiabetic control mice (n = 9) (6.4 ± 1.4 versus 2.6 ± 0.44; P = 0.04). The aortic tissue of diabetic apoE-null mice had increased AGE accumulation (Fig. 2*b* and *c*) compared with that of controls (Fig. 2*d* and *e*), as shown by affinity-purified anti-AGE IgG (ref. 12). Consistent with previous observations in human vasculature, in which enhanced expression of vascular RAGE was associated with an AGE-enriched vascular milieu[22], aortae from diabetic apoE-null mice had considerably increased RAGE expression (Fig. 2*h–j*) compared with that of nondiabetic control mice (Fig. 2*k–l*), by immunohistochemistry.

Suppression of accelerated diabetic atherosclerosis

Soluble RAGE (sRAGE), the extracellular two-thirds of the receptor, binds up AGEs and interferes with their ability to bind and activate cellular RAGE (ref. 12). Because sRAGE reversed hyperpermeability in diabetic rats[13], we investigated whether chronic administration of sRAGE, initiated immediately after documentation of stz-induced diabetes, would suppress accelerated atherosclerosis. Because the half-life for elimination of sRAGE in diabetic rodents was about 22 hours[13], we administered sRAGE once daily (intraperitoneally) to diabetic apoE-null and euglycemic apoE-null mice. Compared with diabetic mice receiving mouse serum albumin (MSA), those treated with sRAGE had dose-dependent suppression of accelerated atherosclerosis (Fig. 3*a*). Indeed, at a dose of sRAGE of 40 μg/day, the mean atherosclerotic lesion area was not significantly different from that in age-matched nondiabetic apoE-null mice treated with MSA. Dose-dependent decreases in visible atherosclerotic plaque in lesion-prone areas were seen by gross inspection of the aortae of diabetic mice treated with sRAGE (Fig. 1*b*, 3 μg/day; *c*, 20 μg/day; and *d*, 40 μg/day]). Total lesion number and complexity were also decreased in diabetic mice treated with sRAGE (Fig. 3*b* and *c*, respectively).

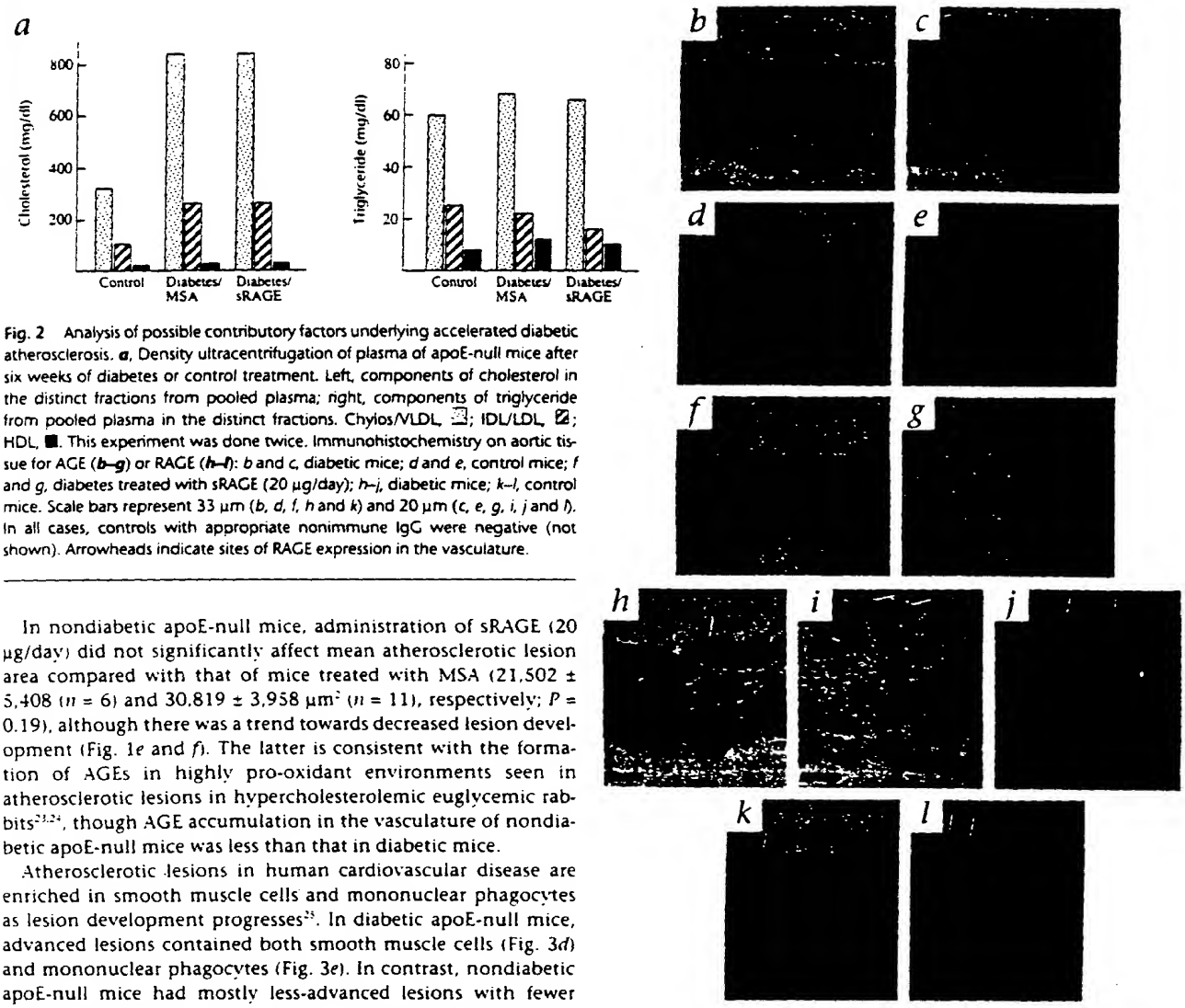

Fig. 2 Analysis of possible contributory factors underlying accelerated diabetic atherosclerosis. *a*, Density ultracentrifugation of plasma of apoE-null mice after six weeks of diabetes or control treatment. Left, components of cholesterol in the distinct fractions from pooled plasma; right, components of triglyceride from pooled plasma in the distinct fractions. Chylos/VLDL, ▨; IDL/LDL, ▨; HDL, ■. This experiment was done twice. Immunohistochemistry on aortic tissue for AGE (*b–g*) or RAGE (*h–l*): *b* and *c*, diabetic mice; *d* and *e*, control mice; *f* and *g*, diabetes treated with sRAGE (20 μg/day); *h–j*, diabetic mice; *k–l*, control mice. Scale bars represent 33 μm (*b*, *d*, *f*, *h* and *k*) and 20 μm (*c*, *e*, *g*, *i*, *j* and *l*). In all cases, controls with appropriate nonimmune IgG were negative (not shown). Arrowheads indicate sites of RAGE expression in the vasculature.

In nondiabetic apoE-null mice, administration of sRAGE (20 μg/day) did not significantly affect mean atherosclerotic lesion area compared with that of mice treated with MSA (21,502 ± 5,408 (n = 6) and 30,819 ± 3,958 μm² (n = 11), respectively; P = 0.19), although there was a trend towards decreased lesion development (Fig. 1*e* and *f*). The latter is consistent with the formation of AGEs in highly pro-oxidant environments seen in atherosclerotic lesions in hypercholesterolemic euglycemic rabbits[23,24], though AGE accumulation in the vasculature of nondiabetic apoE-null mice was less than that in diabetic mice.

Atherosclerotic lesions in human cardiovascular disease are enriched in smooth muscle cells and mononuclear phagocytes as lesion development progresses[25]. In diabetic apoE-null mice, advanced lesions contained both smooth muscle cells (Fig. 3*d*) and mononuclear phagocytes (Fig. 3*e*). In contrast, nondiabetic apoE-null mice had mostly less-advanced lesions with fewer numbers of these cells (Fig. 3*f* and *g*). Diabetic mice treated with sRAGE (20 μg/day) had diminished numbers of both smooth muscle cells and mononuclear phagocytes (Fig. 3*h* and *i*, respectively), consistent with decreased activation of vascular and inflammatory cell in the presence of sRAGE.

Mechanisms underlying beneficial effects of sRAGE

To determine the mechanisms underlying the beneficial effects of sRAGE, we sought to determine if standard risk factors for accelerated vascular disease had been modified. Treatment of diabetic mice with sRAGE (n = 11) did not affect the persistence of hyperglycemia (mean glycosylated hemoglobin, 11.5 ± 0.73%; P = 0.56 compared with diabetic mice treated with MSA) or level of insulinemia (n = 3) (serum insulin, 0.63 ± 0.03 ng/ml; P = 0.79 compared with diabetic mice treated with MSA). Total cholesterol and triglyceride were similarly unaffected by treatment with sRAGE (plasma cholesterol, 1,438 ± 86 mg/dl; P = 0.64 compared to diabetic mice treated with MSA; and plasma triglyceride, 150 ± 8 mg/dl; P = 0.49 compared to diabetic mice treated with MSA). Lipid particle composition was also not changed in diabetic apoE-null mice treated with sRAGE (20 μg/day) compared to those receiving MSA, as demonstrated by ultracentrifugation (Fig. 2*a*) and FPLC separation (data not shown). These data indicate that the beneficial effects of sRAGE seemed both independent of both glycemia and lipid; thus, factors unique to the diabetic environment were favorably affected.

One mechanism by which sRAGE might exert its effects is by binding AGEs and interfering with their ability to activate cell surface receptors[12,13]. A consequence of AGE interaction with sRAGE might be the formation of circulating complexes and, subsequently, enhanced removal of AGEs from diabetic tissues. Immunoprecipitation of plasma with anti-RAGE IgG, followed by SDS–PAGE and immunoblotting of precipitated material with anti-RAGE IgG and anti-AGE IgG demonstrated the presence of molecular species immunoreactive with both antibodies (Fig. 4*a*). To determine whether AGE deposition is affected in diabetic apoE-null mice treated with sRAGE, ELISA was done. Levels of kidney tissue AGE in diabetic mice were suppressed by administration of sRAGE to the levels in nondiabetic mice, in a dose-dependent manner (11.85 ± 1.1 ng/ml AGE BSA equivalents in diabetic mice treated with MSA; 8.97 ±

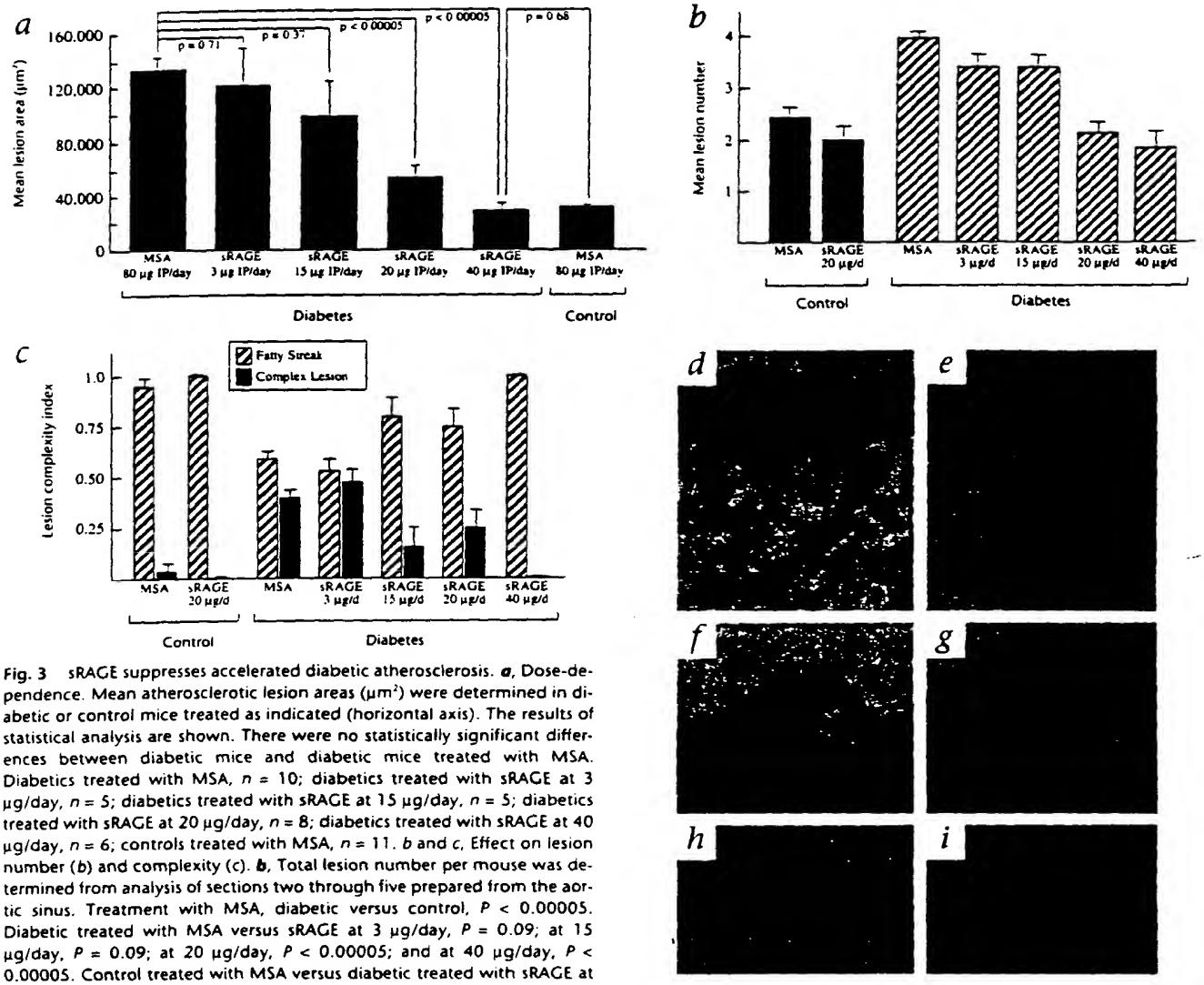

Fig. 3 sRAGE suppresses accelerated diabetic atherosclerosis. ***a***, Dose-dependence. Mean atherosclerotic lesion areas (μm^2) were determined in diabetic or control mice treated as indicated (horizontal axis). The results of statistical analysis are shown. There were no statistically significant differences between diabetic mice and diabetic mice treated with MSA. Diabetics treated with MSA, $n = 10$; diabetics treated with sRAGE at 3 μg/day, $n = 5$; diabetics treated with sRAGE at 15 μg/day, $n = 5$; diabetics treated with sRAGE at 20 μg/day, $n = 8$; diabetics treated with sRAGE at 40 μg/day, $n = 6$; controls treated with MSA, $n = 11$. *b* and *c*, Effect on lesion number (*b*) and complexity (c). ***b***, Total lesion number per mouse was determined from analysis of sections two through five prepared from the aortic sinus. Treatment with MSA, diabetic versus control, $P < 0.00005$. Diabetic treated with MSA versus sRAGE at 3 μg/day, $P = 0.09$; at 15 μg/day, $P = 0.09$; at 20 μg/day, $P < 0.00005$; and at 40 μg/day, $P < 0.00005$. Control treated with MSA versus diabetic treated with sRAGE at 20 μg/day, $P = 0.30$; and at 40 μg/day, $P = 0.12$. Controls treated with MSA, $n = 11$; controls treated with sRAGE at 20 μg/day, $n = 6$; diabetics treated with MSA, $n = 10$; diabetics treated with sRAGE at 3 μg/day, $n = 5$; diabetics treated with sRAGE at 15 μg/day, $n = 5$; diabetics treated with sRAGE at 20 μg/day, $n = 8$; diabetics treated with sRAGE at 40 μg/day, $n = 6$. ***c***, Lesion complexity index was calculated from the ratio of fatty streak (FS) divided by the total lesion number, or complex (C) lesion (defined by presence of cholesterol clefts, necrosis or fibrous cap formation) divided by total lesion number . Therefore, the sum of the ratio of FS/total and C/total is one. For fatty streak index: Diabetic treated with MSA versus sRAGE at 3 or 15 μg/day, P = not significant; at 20 or 40 μg/day, $P < 0.0005$. For complex lesion index: Diabetic treated with MSA versus sRAGE at 3 or 1: μg/day, P = not significant; at 20 μg/day, $P = 0.19$; and at 40 μg/day, $P = 0.00005$. ***d–l***, Cellular composition of atherosclerotic lesions. Section from the aortic sinus were fixed and subjected to immunohistochemistr for α-smooth muscle actin (*d*, *f* and *h*) and CD68 (*e*, *g* and *i*). *d* and *e*, diabetic treated with MSA; *f* and *g*, control; *h* and *i*, diabetic treated with sRAGE at 20 μg/day. In all cases, controls with appropriate nonimmun IgG were negative (not shown). Scale bar represents 33 μm.

1.7 ng/ml AGE BSA equivalents in diabetic mice treated with 3 or 15 μg sRAGE per day (pooled; $n = 6$); $P = 0.18$ compared with diabetic mice treated with MSA; and 6.06 ± 1.3 ng/ml AGE BSA equivalents in diabetic mice treated with 20 or 40 μg/day sRAGE (pooled; $n = 5$); $P = 0.006$ compared with diabetic mice treated with MSA). Similar results were obtained by analysis of plasma AGEs. Levels of plasma AGE were 6.4 ± 1.4 ng/ml AGE BSA equivalents in diabetic mice treated with MSA; in contrast, in diabetic mice treated with sRAGE (20 μg/day), levels of plasma AGE were significantly reduced (2.7 ± 0.64 ng/ml AGE BSA equivalents; $n = 3$; $P = 0.04$ compared with diabetic mice treated with MSA). These data indicate sRAGE is a molecular 'sponge' for AGEs, and are consistent with the hypothesis that sRAGE binds AGEs, thereby preventing interaction with cell surface RAGE. A consequence of suppressed activation of cell surface RAGE is inhibition of receptor-dependent signal transduction mechanisms[26,27]. Such inhibition might diminish oxidant stress, another factor in the diabetic milieu that may accelerate AGE formation. Although experiments are underway to determine the mechanisms underlying diminished AGE deposition in sRAGE-treated mice, study of LDL isolated from sRAGE-treated mice provided support for

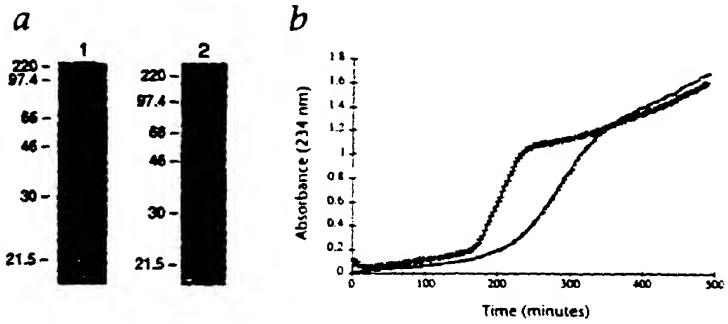

Fig. 4 Analysis of potential contributory factors underlying the beneficial effects of sRAGE in suppressing accelerated diabetic atherosclerosis. *a*, Plasma from mice treated with sRAGE was immunoprecipitated with anti-RAGE IgG and immunoblotted with anti-RAGE IgG (68 μg/ml; lane 1) or affinity-purified anti-AGE IgG (4.5 μg/ml; lane 2). Similar results were obtained by immunoprecipitation with anti-AGE IgG; however, neither RAGE- nor AGE-immunoreactive material was retrieved by immunoprecipitation using nonimmune IgG (not shown). Left margins, positions of molecular weight markers. *b*, Conjugated dienes. LDL was isolated from pooled mouse plasma (5 mice per group) by ultracentrifugation and copper-induced oxidizability determined. Line with hashmark, diabetic treated with MSA; line with solid square, diabetic treated with sRAGE. This experiment was done twice.

the idea of a diminished pro-oxidant state in the presence of soluble receptor. Susceptibility to copper-induced oxidation of LDL (refs. 28,29) retrieved from diabetic apoE-null mice treated with sRAGE (20 μg/day) was diminished compared with that of diabetic mice treated with MSA; mean lag-time to formation of conjugated dienes was 185.3 versus 135.3 minutes (Fig. 4*b*).

Treatment of diabetic mice with sRAGE did not seem to produce adverse effects, although this was a short-term study. Diabetic mice treated with sRAGE and MSA had similar trends in weight gain, food/water intake and activity (data not shown). Because there are small amounts of sRAGE (about 1 ng/ml) in the plasma of euglycemic mice (as well as that of rats, humans and cows) under homeostatic conditions, it was not surprising that sRAGE did not elicit antibody formation, based on serial analyses of sera after as much as six weeks of daily parenteral treatment with sRAGE (data not shown).

Discussion

Our studies demonstrate a model of accelerated, complex atherosclerotic lesion development in diabetic mice. As in human atherosclerosis, especially in the context of diabetes (in which aggressive formation of complex lesions is characteristic), our model provides a setting in which to study the many factors involved in the increased incidence and severity of macrovascular disease associated with diabetes. Indeed, as many studies have not conclusively described a direct role for hyperglycemia in accelerated diabetic vascular disease (especially in male subjects)[30,31], this model may be a useful tool to study relevant factors in diabetes in a controlled environment.

Here, an important control for potential toxic effects of stz (ref. 18) included analysis of apoE-null mice that received the same dose and lot of drug as other mice studied, but did not develop hyperglycemia (≤5% of mice); these mice did not develop accelerated atherosclerosis (data not shown), consistent with absence of a direct effect of stz on the development of vascular lesions. Indeed, stz did not affect hepatic lipid content in C57BL/6 mice[15]. In support of the apparent lack of generalized adverse effects of stz, levels of serum creatinine and alanine aminotransferase (ALT) were not different for control mice, diabetic mice treated with MSA and diabetic mice treated with sRAGE using this dose and schedule of stz (data not shown). Furthermore, recent studies from our laboratory have shown accelerated atherosclerosis develops in hyperglycemic heterozygote apo-E null mice bred into the homozygotic diabetic (db+/db+) background, compared with that in nondiabetic counterparts, thus providing a model of accelerated vascular disease in a model of type 2 diabetes in which insulin resistance underlies hyperglycemia and no stz is employed (data not shown).

Nakashima *et al.* described advanced lesions developed at 15 weeks of age in apoE-null mice[20], similar to the age of the mice studied here (13–14 weeks). The mice used by Nakashima *et al.* were second- or third-generation hybrids (129ola × C57BL/6 or 129ola × BALB/c). In contrast, the mice here were back-crossed ten generations into the C57BL/6 background. Such genetic heterogeneity probably accounted for these differences.

ApoE-null mice developed increased levels of cholesterol (VLDL and LDL), without alterations in triglyceride, when diabetes was induced. This differs from what usually occurs in human diabetes. Our model, nevertheless, provides an important new approach to determine the contribution of both lipid-mediated and lipid-independent factors in diabetic atherosclerosis. In that context, the beneficial effects of sRAGE in apoE-null mice indicate an important role for the interaction of AGEs with RAGE in the pathogenesis of accelerated atherosclerosis. Because the administration of sRAGE fully suppressed accelerated diabetic atherosclerosis, these data support the hypothesis that sRAGE may also prevent interaction of AGEs with other putative AGE-binding proteins, such as macrophage scavenger receptor, which was recently implicated in the pathogenesis of atherosclerosis in nondiabetic animals[32,33].

These data indicate that sRAGE may be not only a new tool to describe the molecular mechanisms involved in accelerated diabetic vascular disease, but also a prototypic structure for the design of agents to prevent macrovascular complications in diabetes. Indeed, as strict management of hyperglycemia in the Diabetes Control and Complications Trial Research Group trials produced equivocal results in protection against macrovascular complications[4], such an agent would fill an important gap in current therapy.

Methods

Mice deficient in apolipoprotein E and induction of diabetes. Male homozygous apoE-null mice (back crossed ten times into the C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, Maine) and maintained on a twelve-hour light–dark cycle in a non-pathogen-free environment on normal chow (4.5% fat [9% of calories]). At six weeks of age, mice were treated with six daily intraperitoneal injections of streptozotocin (stz; Sigma), a dose of 55–65 mg/kg in citrate buffer (0.05 M; pH 4.5). Control mice received citrate buffer. Serum glucose was measured from tail vein blood using the glucose oxidase method (Sigma) in nonfasted mice. Glycosylated hemoglobin in lysates of red blood cells was determined by affinity chromatography (Pierce, Rockford, Illinois). Insulin levels were determined using a radioimmunoassay system (Linco, Balwin,

Missouri). Markers of hepatic and renal function were done by the University of Miami Pathologic Reference Services.

Soluble mouse RAGE. We prepared sRAGE in a baculovirus expression system using Sf9 cells (Clontech, Palo Alto, California; Invitrogen, Carlsbad, California). Serum-free medium containing sRAGE was subjected to FPLC Mono S for purification (Pharmacia). Purified murine sRAGE (a single-band of about 40 kDa, by Coomassie-stained SDS–PAGE) was dialyzed against phosphate-buffered saline (PBS); made free of detectable endotoxin, based on the Limulus amebocyte assay (Sigma) after passage onto Detox-igel columns (Pierce, Rockford, Illinois); and sterile-filtered (0.2 μm). Mouse serum albumin (MSA) (Sigma) was used as a control at equimolar concentrations compared with sRAGE.

Quantitation of atherosclerotic lesion area. After the mice were killed, their blood was removed from the inferior vena cava into EDTA (final concentration, 0.05M), and plasma/red blood cells were stored for analysis. For each mouse, the aorta was perfused gently with PBS in a retrograde manner and the heart was then removed and stored in buffered formalin (10%) (ref. 21). Cryostat sections were prepared and embedded in gelatin (25%). Serial sections, 10 μm in thickness, were cut from the level of the aortic valve leaflets up to about 480 μm above the leaflets in the aortic sinus; alternate sections were retrieved and placed onto gelatin-coated slides (5%); four sections were placed onto each slide for a total of five slides. Sections were then stained and counterstained with oil red O and hematoxylin/light green. Atherosclerotic lesion areas were quantified on one section from each slide (beginning at the site where three distinct valves first appear) using a Zeiss microscope and image analysis system (Media Cybernetics, Silver Springs, Maryland); mean lesion area from slides two through five is reported. Two of the investigators, 'blinded' to the experimental conditions, analyzed the slides and performed the morphometric analysis.

Lipoprotein analysis. Levels of total cholesterol and triglyceride were determined in fasted mice (no food from 7 AM to 11 AM) using chromogenic assays (Sigma and Boehringer). Fast Performance Liquid Chromatography (FPLC; Pharmacia) was done using two Superose 6 columns (Pharmacia) run in series onto which was applied pooled, sterile-filtered mouse plasma (five mice per assay). Lipid components in pooled mouse plasma were separated by sequential density ultracentrifugation as described[34].

Determination of conjugated dienes. Assays to measure the formation of conjugated dienes (copper-induced oxidizability of LDL) were done as described[35,36]. LDL was isolated from pooled mouse plasma and dialyzed in the dark for 24 h at 4 °C against 3 liters of PBS containing EDTA (10 μM). Buffers were made oxygen-free by vacuum degassing followed by purging with nitrogen, and sterilized by passage through filters with 0.45 μm pores. *In vitro* oxidation was done using dialyzed LDL containing EDTA (10 μM), adjusted to 1.0 mg LDL protein/ml, diluted immediately before the start of the oxidation with EDTA-free phosphate buffer to a final concentration of 0.10 mg LDL protein/ml (thus reducing the final concentration of EDTA to 1 μM). Oxidation was initiated by the addition of a freshly-prepared $CuCl_2$–H_2O solution (final concentration 15 μM). The kinetics of LDL oxidation were determined by monitoring the change in the 234-nm absorbance at 30 °C in an UltraSpec Plus spectrophotomer (Pharmacia). The lag-time to diene production was determined graphically as the time point at which the tangent to the curve during the maximum slope of the propagation phase intercepted the time axis.

Determination of AGE content. Affinity-purified anti-AGE IgG was prepared as described[12] and used in an ELISA with known concentrations of AGE-bovine serum albumin as standard (limit of detection, 0.06 ng/ml AGE albumin equivalents) (ref. 11). Kidney tissue was subjected to extraction of acid-soluble material by exposure to HCl (6N) at 110 °C for 16 hours. Supernatant was retrieved by centrifugation and analyzed for AGE content.

Immunohistochemistry. Samples were analyzed for AGE and RAGE by immunohistochemistry as described[12,22]. Final concentrations of the respective antibodies were 8 μg/ml and 85 μg/ml. For detection of murine macrophages and smooth muscle cells, anti-CD68 IgG (final concentration, 210 μg/ml; Dako, Carpinteria, California) and anti-alpha smooth muscle actin (final concentration, 152 μg/ml; Dako, Carpinteria, California) w used according to the manufacturer's instructions (Dako and Sigma). I cases, the same species of nonimmune IgG was used as a control (Sign

Immunoprecipitation. Plasma of diabetic mice treated with sRAGE (20 for six weeks) was immunoprecipitated. Plasma was pre-cleared with c trol rabbit IgG and immunoprecipitation was done using either monos cific, polyclonal rabbit anti-mouse RAGE, affinity-purified rabbit anti-A IgG or nonimmune IgG. IgG-bound material was then retrieved using p tein A agarose (Pierce, Rockford, Illinois), eluted with nonreducing Laem buffer and separated by SDS–PAGE (10%). Immunoblotting, after el trophoretic transfer of protein onto nitrocellulose (Bio-Rad), was done described[7,8].

Statistical analysis. All data are reported as mean ± standard error of the mea Data were analyzed by ANOVA and, as indicated, subject to post-hoc compa isons using two-tailed *t*-test. Values considered significant were $P < 0.05$.

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